REMARKS

Claim 1 has been amended. Claims 1-6 and 8-9 are now pending in this application. Support for the amendments is found in the existing claims and the specification as discussed below. Accordingly, the amendments do not constitute the addition of new matter. Applicant respectfully requests the entry of the amendments and reconsideration of the application in view of the amendments and the following remarks.

Rejection under 35 U.S.C. § 103(a)

Claims 1-6 and 8-9 remain rejected under 35 U.S.C. § 103(a) as being unpatentable over Sode (WO 2002/36779, publ. May 10, 2002) in view of Herbaud, et al. (BBA 1481 (1) :18, 2000) as evidenced by Arslan, et al. (BBRC 251: 744, 1998).

The Office Action states that the claims do not recite comparison to a wild or recombinant bacterial strain which do not have the genes of a ccm operon linked to a promoter (Office Action, page 4, first paragraph). In response, Claim 1 has been amended to replace the "whereby" clause with "wherein the expression of a cytochrome *c* maturation system (ccm) and glucose dehydrogenase is enhanced compared to a wild strain or unmodified strain of *Escherichia* bacteria". Support for the amendment is found in the present specification at page 9, lines 2-5. Claim 1 has been further amended to limit the β-subunit of glucose dehydrogenase to the KS1 strain of *Burkhorderia cepacia*.

The Examiner refers to Herbaud, et al. at page 18 col. 2 as basis for the previous assertion that Herbaud came to the same conclusion as the inventors that co-expression of ccm with GDH causes improved expression. However, Herbaud, et al. are silent regarding GDH. In the section referred to by the Examiner, it is merely stated that "it has been shown that when the *ccm* genes are provided on a plasmid together with the structural gene for a mono- and a diheme *c*-type cytochrome, the cytochrome maturation occurs and seems to be increased". As stated previously, this would be expected as cytochrome c and the ccm genes are found together in vivo. However, although the β-subunit of GDH of *Burkhorderia cepacia* KS1 strain is a c-type cytochrome, it is unexpected that co-expression of the ccm genes with GDH would result in greatly enhanced expression of GDH as the ccm genes are **not** found together with GDH in vivo.

Furthermore, the β subunit of GDH, which has the cytochrome *c* activity, is clearly a different protein from the cytochrome *c* of Herbaud as the molecular weight is 43 kDa by SDS-page (see paragraph 0181 of US 2004/0023330) whereas cytochrome c_3 of Herbaud has molecular weight of 13 kD (page 20, col. 2, line 5). In addition, GDH includes α and γ subunits.

The Office Action states that "the structure of the claimed bacterium is suggested by the prior art and the examiner has made the prima facie case" (Office Action, page 5, second paragraph). The Examiner asserts that "Herbaud came to the same conclusion as the inventors that co-expression of ccm with GDH causes improved expression,...", but there is no teaching in Herbaud on GDH. The Office Action does not address this deficiency.

Applicants provide herewith additional evidence that the levels of expression of GDH in an *Escherichia* bacterium comprising "genes of a ccm operon operably linked to a promoter" were unexpected.

The Examiner's attention is directed to Sinha, et al. FEMS Microbiology Letters 161: 1, 1998 (Attachment A). The Abstract of Sinha, et al. discloses that elevated production of c-type cytochromes was observed in a strain of *Hydrogenobacter thermophilus* that lacked a complete set of ccm genes. The Abstract states that "...elevated production of c-type cytochromes is not a consequence of high activity of ccm genes but rather an enhanced ability to supply haem...". Accordingly, Sinha, et al. teach away from the claimed invention.

The teaching of Sinha, et al. is consistent with the teaching of Herbaud, et al. Herbaud, et al. also teach that cytochrome c3 production was increased by 10% when 0.1 mM δ-aminolevulinic acid, a heme precursor, was added to the liquid media under aerobic conditions (see Herbaud, et al., page 21, col. 2, lines 6-8). Accordingly, Herbaud, et al., like Sinha, et al. teach that production of a c-type cytochrome is improved when ability to supply heme is enhanced. Based upon Sinha, et al., one of ordinary skill in the art would not combine genes of a ccm operon and a glucose dehydrogenase and expect to obtain enhanced levels of GDH. Based upon Sinha, et al. (and Herbaud, et al.) one would provide δ-aminolevulinic acid or other means to increase production of heme in order to increase cytochrome c production.

While Sode teaches constitutive expression of GDH and Herbaud, et al. teach the ccm system in combination with a cytochrome c, there was no apparent reason to combine GDH and ccm genes to enhance GDH expression. Indeed, Sinha, et al. ***teach away*** from the combination.

While the references taken together teach the claim elements, the combination is non-obvious in view of the teaching away reference submitted herewith (Sinha) and the unexpected high expression of GDH which has been discussed previously and also below.

Applicants point out that when the αβγ subunits of glucose dehydrogenase are expressed without the ccm system, expression is still less than the non-recombinant KS1 strain as shown in the present specification at page 20, second full paragraph. Sode (WO 2002/36779) teaches the same plasmid but without the β-subunit (see Examples 11 and 12 of US 2004/0023330, English language equivalent to WO 2002/36779). Accordingly, expression is likely to be even lower than in the present specification which teaches expression of αβγ subunits.

Furthermore, even if Sode (WO 2002/36779) were to include the β-subunit of GDH, it would be expected that expression of GDH would still be less than expression of GDH from non-recombinant *Burkhorderia cepacia* KS1 in view of the data in the specification. By including the ccm genes with the αβγ subunits of glucose dehydrogenase (JM109/pTrc99Aγαβ, pBBJPccm), expression was elevated 100 times over JM109/pTrc99Aγαβ. In view of the observation that expression of GDH was lower than that of *Burkhorderia cepacia* KS1 when the β-subunit was co-expressed with the α-subunit and γ-subunit without enhancing the ccm genes, one of ordinary skill in the art would not have expected that the GDH expression would increase 100 times by inclusion of ccm genes.

Even if the Examiner's position is accepted, that Herbaud teaches an increase in cytochrome c in the presence of ccm genes of 10% which means that GDH activity will also increase in presence of ccm genes (see Office Action, page 6, first paragraph referring to Herbaud, page 21, col. 2 ,lines 3-5), this result contrasts with the enhancement observed by Applicants which is ***100 times*** the activity observed without the ccm genes (comparing JM109/pTrc99Aγαβ to JM109/pTrc99Aγαβ, pBBJPccm, present specification, page 20). Based upon Sode and Herbaud, one would expect an increase in cytochrome c activity of 10%, not an increase in GDH activity 100 times the level without ccm genes expressed. Accordingly, the level of increased expression, which is a characteristic of the claimed bacterial system, was unexpected.

The Examiner basis for discounting previous arguments regarding the inability of Arslan, et al. to stimulate cytochrome c-550 production with pEC86 (Arslan, et al. page 747, col. 1, last

paragraph) (Office Action, page 6, paragraph 20) is not clear as, contrary to the assertion of the Examiner, pEC86 ***was*** used as source of ccm genes and in any case, pEC86 is a convenient source for ccm genes but claim 1 does not require pEC86. Reconsideration is requested.

The Examiner also states on page 10, paragraph 2 of the Office Action that the "skilled artisan would have had a reasonable expectation of success in combining the teaching of Sode and Herbaud, et al. because each of these teachings generated enhancement of the ccm system". Applicants assume that the Examiner intended Arslan and Herbaud, not Sode, as Sode does not teach anything regarding ccm.

In view of Applicants' amendments and arguments, reconsideration and withdrawal of the above ground of rejection is respectfully requested.

Rejection under 35 U.S.C. § 112, second paragraph

Claims 1-6 and 8-9 are rejected under 35 U.S.C. § 112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The Examiner states that the previous amendment to "...thereby enhancing the expression of a cytochrome *c* maturation (ccm) system, and glucose dehydrogenase" is indefinite as the specification does not specifically mention "enhancing expression of glucose dehydrogenase" (Office Action, page 11, last paragraph). The Examiner states that "the specification indicates 'expression of the ccm system is enhanced' means that the expression is enhanced compared with that in a wild strain or unmodified strain of *Escherichia* bacteria" (Office Action at page 11-12, referring to specification at page 9, lines 2-5). Accordingly, claim 1 has been amended to incorporate this language as supported by the specification at page 9, lines 2-5. The previous amendment has been deleted.

In view of Applicants' amendment and remarks, reconsideration and withdrawal of the above ground of rejection is respectfully requested.

Rejection under 35 U.S.C. § 112, second paragraph

Claims 1-6 and 8-9 are rejected under 35 U.S.C. § 112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The Examiner states that the terms "improving" and "high" are relative terms which are indefinite. This language has been deleted in favor of the present amendment to claim 1 discussed above. Accordingly, this rejection may be withdrawn.

No Disclaimers or Disavowals

Although the present communication may include alterations to the application or claims, or characterizations of claim scope or referenced art, Applicant is not conceding in this application that previously pending claims are not patentable over the cited references. Rather, any alterations or characterizations are being made to facilitate expeditious prosecution of this application. Applicant reserves the right to pursue at a later date any previously pending or other broader or narrower claims that capture any subject matter supported by the present disclosure, including subject matter found to be specifically disclaimed herein or by any prior prosecution. Accordingly, reviewers of this or any parent, child or related prosecution history shall not reasonably infer that Applicant has made any disclaimers or disavowals of any subject matter supported by the present application.

CONCLUSION

In view of Applicants' amendments to the claims and the foregoing Remarks, it is respectfully submitted that the present application is in condition for allowance. Should the Examiner have any remaining concerns which might prevent the prompt allowance of the application, the Examiner is respectfully invited to contact the undersigned at the telephone number appearing below.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: May 6, 2009

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FEMS Microbiology Letters 161 (1998) 1–6

FEMS MICROBIOLOGY LETTERS

An *Escherichia coli ccm* (cytochrome *c* maturation) deletion strain substantially expresses *Hydrogenobacter thermophilus* cytochrome c_{552} in the cytoplasm: availability of haem influences cytochrome c_{552} maturation

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Abstract

The maturation of *Hydrogenobacter thermophilus* cytochrome c_{552} in the cytoplasm of *Escherichia coli* is unique among bacterial *c*-type cytochromes. It is now shown to be matured in a strain lacking the whole set of *ccm* (cytochrome *c* maturation) genes that are normally required for *c*-type cytochrome biogenesis in *E. coli*. As this cytochrome is thermostable we propose that the apocytochrome c_{552} has sufficient tertiary structure to allow the haem to slot into its binding pocket, which in turn triggers the spontaneous covalent attachment between apocytochrome c_{552} and haem. The *ccm* deletion strain of *E. coli*, derived from a strain that synthesizes elevated levels of endogenous *c*-type cytochromes, also produces larger amounts of cytoplasmic *H. thermophilus* cytochrome c_{552} than a reference strain. This implies that elevated production of *c*-type cytochromes is not a consequence of high activity of *ccm* genes but rather an enhanced ability to supply haem, a view that is supported by the increase in thermophilic cytochrome c_{552} biogenesis that occurs in a reference strain following supplementation of growth media with δ-aminolevulinic acid. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V.

Keywords: *Hydrogenobacter thermophilus*; Cytochrome *c* biogenesis; Haem; δ-Aminolevulinic acid

1. Introduction

C-type cytochrome biogenesis in bacteria involves a post translational pathway for the conversion of pre-apocytochrome *c* into the mature holocytochrome *c*. *C*-type cytochromes differ from other classes of cytochromes on account of the mechanism of haem attachment to the cytochrome *c* polypeptide. The haem moiety is attached to the polypeptide by thioether bonds between the two haem vinyl groups and the thiol groups of two cysteine residues of the conserved motif Cys-X-Y-Cys-His. Several lines of evidence suggest that this covalent attachment takes place in the periplasm and a relatively detailed model has been proposed [1] for the biogenesis of *c*-type cytochromes in bacteria. This model is in agreement with the results of studies on *Paracoc-*

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cus denitrificans cytochrome *c* biogenesis indicating that the maturation of *c*-type cytochrome in bacteria takes place in the periplasm [2]. Genes directly involved in the biogenesis of *c*-type cytochromes have been found in many Gram-negative organisms such as *Rhodobacter casulatus* [3,4], *Bradyrhizobtum japonicum* [5,6], *Escherichia coli* [7] and *P. denitrificans* [8–10]. Some of the components involved in the biogenesis of *c*-type cytochromes are thought to be periplasmic or attached to the cytoplasmic membrane with their functional domain facing towards the periplasm [11–13]. It has been shown that the holoform of *P. denitrificans* cytochrome c_{550} is only found in either *P. denitrificans* or *E. coli* when the polypeptide, expressed from a plasmid, is targeted to the periplasm by its signal sequence [14]. Removal of the latter sequence results in the appearance of an apoform of the protein in the cytoplasm of both the organisms [14]. Cytochrome c_{552} from a thermophilic bacterium, *Hydrogenobacter thermophilus*, is the only cytochrome *c* which has been shown to be matured in the cytoplasm of *E. coli*; this occurs when it is expressed from a construct which lacks the coding region for its signal sequence [14]. Furthermore, it was shown that this cytochrome c_{552} was also expressed in an *E. coli* mutant strain lacking a gene, *dipZ*, coding for a disulfide isomerase or thioredoxin like protein [15], essential for other normal *c*-type cytochrome maturation [16]. Thus, it was suggested that the cytoplasmic maturation of this thermophilic cytochrome c_{552} does not need any enzymatic assistance [14,15]. However, this suggestion, with its very significant implication that covalent attachment of haem to the polypeptide of apocytochromes *c* can be uncatalysed, relies on the supposition that none of the other genes required for *c*-type cytochrome biogenesis in *E. coli* (*ccm* (cytochrome *c* maturation) genes) have any unsuspected role in this cytoplasmic synthesis of *H. thermophilus* cytochrome c_{552}, perhaps through currently unrecognized activities of cytoplasmic facing regions of some of the gene products. The present paper, therefore, addresses the important question as to whether the expression of the cytoplasmic cytochrome c_{552} continues in a strain of *E. coli* from which the *ccm* genes are deleted. The *ccm* deletion strain used is derived from a parent strain, JCB712, which produces, for unknown reasons, elevated levels of endogenous, periplasmic facing, *c*-type cytochromes [17]. Thus it was of considerable interest to determine whether this strain would also produce elevated levels of cytoplasmic cytochrome c_{552}. The outcome of this pair of experiments was thus expected to establish whether the activity of the *ccm* gene products might be responsible for the relatively high extent of endogenous cytochrome *c* biogenesis in *E. coli* JCB712, and if not, whether another factor, for example supply of haem, might be important.

2. Materials and methods

2.1. Bacterial strains and plasmids

The strains and plasmids used are listed in Table 1.

2.2. Preparation and analysis of normalized cell extracts for comparison of expression of cytochrome c_{552}

The normalized crude extracts were prepared by growing the cells in LB, aerobically, at 37°C until a culture had reached an OD of 1.5 at 580 nm. The same OD was obtained for each cell culture so that comparison of the expression in different strains of cytochrome c_{552} could be made. The cell pellet, collected by centrifugation at 12000 rpm at 4°C, was resuspended in GTE (50 mM glucose, 25 mM Tris-HCl pH 7.5, 10 mM EDTA) and the cell walls were broken by freezing and thawing. The plasma membrane was removed by sonication and centrifugation. This resulting supernatant was then used as the crude extract. The amount of total cell protein in the crude extract was measured, using a protein assay kit from Biorad, and equal amounts of protein were loaded onto 15% SDS-PAGE gels for the comparisons of expression. Changes in the expression of cytochrome c_{552} were also determined for each sample by spectrophotometry. The crude extracts were suitably diluted, reduced with dithionite and the absorbance at 552 nm was measured on a Perkin Elmer UV/Vis spectrophotometer. SDS-PAGE and other molecular biology techniques were followed as described by Sambrook et al. [18]. Staining for the detection of covalently attached haem was done as

described by Goodhew et al. [19]. In some experiments the haem precursor, δ-aminolevulinic acid, was added to the growth medium, usually at a final concentration of 0.1 mM. Recombinant *H. thermophilus* cytochrome c_{552} was partially purified, so as to provide a standard for SDS-PAGE, by CM-cellulose chromatography of an extract from *E. coli* containing the construct pKHC12. This cytochrome c_{552} is the only cytochrome in such an extract that binds to the cation exchanger and thus sufficient purification for the present purpose was achieved.

3. Results

3.1. Expression of H. thermophilus cytochrome c_{552} in E. coli ccm deletion strain, JCB71202

Expression of *H. thermophilus* cytochrome c_{552} in *E. coli* strain JCB71202 was readily observed from the red colour of the cells, haem staining following SDS-PAGE (Fig. 1) and by spectrophotometry (Fig. 2A). Thus none of the *ccm* genes that are deleted in this strain are required for cytoplasmic synthesis of this cytochrome. JCB712, the parent strain of JCB71202, for unknown reasons synthesizes higher level of endogenous periplasmic or periplasmic facing *c*-type cytochromes than other strains [17,20]. The ready detection of *H. thermophilus* cytochrome c_{552} in the JCB71202 strain suggested that this higher level of expression may also apply to this cytoplasmically expressed protein. To test this proposal the expression of this cytochrome in strains JCB712 and JCB387, a commonly used strain in the studies of cytochrome *c* biogenesis and expressing lower levels

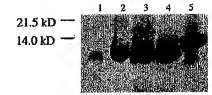

Fig. 1. Haem stained SDS-PAGE gel of the extracts of three strains of *E. coli* expressing *H. thermophilus* cytochrome c_{552} from the construct pKHC12. The normalized crude extracts, ~80 μg protein in each case, were loaded on an SDS-PAGE gel as described in Section 2. Lane 1: Partially purified recombinant cytochrome c_{552} (1 μg protein). Lane 2: Extract of JCB387 expressing cytochrome c_{552}. Lane 3: Extract of JCB712 expressing cytochrome c_{552}. Lane 4: Extract of JCB71202 (*ccm* deleted) expressing cytochrome c_{552}. Lane 5: Horse heart mitochondrial cytochrome *c* (10 μg protein) supplied by Sigma. The molecular masses on the left-hand side correspond to the electrophoresis of two colored marker proteins (rainbow markers supplied by Amersham Life Science), in one lane of the gel but which do not reproduce photographically. Mitochondrial cytochrome c in lane 5 serves as an additional molecular mass marker.

of cytochrome *c* [17,20–22], was examined. Fig. 1 shows that whereas the extent of expression of thermophilic cytochrome c_{552} in JCB712 was comparable with that in JCB71202, a significantly lower level was observed for strain JCB387. Although a normalized amount of crude extract was loaded on SDS-PAGE in each case, the higher level of expression of *H. thermophilus* holocytochrome c_{552} in JCB712 or JCB71202 was also demonstrated by measuring the visible absorbance of this cytochrome (Fig. 2A). This established that the expression of *H. thermophilus* cytochrome c_{552} was many-fold (approximately 10 times) higher in JCB712 strains than in JCB387.

Table 1
E. coli strains and plasmids

Strain or plasmid	Relevant genotype/characteristics	Source [reference]
Strains		
JCB387	*E. coli* RV Δ*nirB*	Griffiths and Cole [21]
JCB712	*pro his trpΔlac*	M. Jones-Mortimer
JCB71202	Δ*ccm*(*A'-H*)::Ω	Grove et al. [17]
Plasmids		
pKHC12	Contains the coding region of cytochrome c_{552} mature protein, with the coding region for the signal sequence deleted.	Sanbongi et al. [27]
pKPHC12ΔSIG	Contains the first ten amino acids of *P. denitrificans* cytochrome c_{550} followed by the coding region for mature protein of cytochrome c_{552}.	Y. Sanbongi

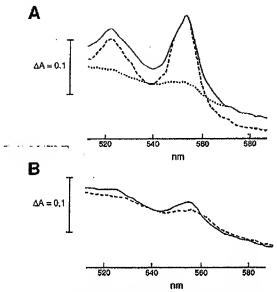

Fig. 2. A: Spectra of reduced *H. thermophilus* cytochrome c_{552} in crude extracts of *E. coli* strains JCB387 (dotted line), JCB712 (line) and JCB71202 (dashed line) expressing cytochrome c_{552} from the construct pKHC12. The crude extracts were prepared as described in Section 2. 0.8 mg protein/ml was used for the analysis after adding sufficient solid sodium dithionite to reduce the samples completely. The spectra were measured against a buffer reference. B: Spectra of reduced cytochrome c_{552} in crude extracts of *E. coli* strain JCB387 expressing cytochrome c_{552} from the construct pKHC12 either without (dashed line), or with (line) 0.1 mM δ-aminolevulinic acid added to the growth medium. Other conditions were as in A.

3.2. The effect of δ-aminolevulinic acid on the expression of cytochrome c_{552}

In principle, an explanation for the higher levels of expression of *c*-type cytochromes in *E. coli* JCB712 strain is that one or more genes (e.g. *ccm*, *dipZ*, *dsbB*) that are required for *c*-type cytochrome biogenesis are expressed to a higher level than in other strains. However, the fact that the cytoplasmically expressed cytochrome c_{552} from *H. thermophilus* is also expressed to a greater extent in both JCB712 and JCB71202 strains, despite its formation being independent of at least the *ccm* and *dipZ* genes, suggests that this cannot be the explanation. *C*-type cytochrome biogenesis requires an appropriate supply of a haem which prompted us to investigate whether the supply of haem precursor, δ-aminolevulinic acid, to the growth media influences the production of the holocytochrome c_{552}. In case of strain JCB387 the inclusion of 0.1 mM δ-aminolevulinic acid in the medium resulted in noticeably redder cells and an increase in the amount of thermophilic cytochrome c_{552} (Fig. 2B). A further increase in the concentration of δ-aminolevulinic acid had no effect (not shown). The dependence on the concentration of δ-aminolevulinic acid is similar to that reported for the expression of other haem proteins in *E. coli* [23]. In contrast, the addition of 0.1 mM δ-aminolevulinic acid to the growth medium for JCB712 strains was without effect on the synthesis of *H. thermophilus* holocytochrome c_{552}. The effect of supplementation of the growth medium with δ-aminolevulinic acid on the expression of cytochrome c_{552} in the strain JCB387 could also be clearly seen when the plasmid pKHC12ΔSig was used. This has the cytochrome c_{552} structural gene sequence preceded by the coding sequence for ten residues from the N-terminus of *P. denitrificans* cytochrome c_{550} protein. A periplasmic targeting sequence is absent, as in pKHC12. *H. thermophilus* holocytochrome c_{552}, extended by ten residues at N-terminus following expression from pKHC12ΔSig, was not readily detectable in the strain JCB387 unless the growth medium was supplemented with δ-aminolevulinic acid. In contrast, this form of *H. thermophilus* cytochrome c_{552} was expressed from the same plasmid at readily detectable levels in the strain JCB712 without supplementation of growth medium with δ-aminolevulinic acid, to an extent approximately equivalent to that found in JCB387 after supplementation with δ-aminolevulinic acid.

4. Discussion

The biogenesis of *H. thermophilus* cytochrome c_{552} in the *ccm* minus background is striking and strengthens our previous hypothesis that its maturation is independent of enzymatic assistance [14]. The observations that δ-aminolevulinic acid increases the expression of cytochrome c_{552} from the strain JCB387 but that there is no effect of δ-aminolevulinic acid on the expression of cytochrome c_{552} from the strain JCB712, lead us to assume that *E. coli* JCB712, and strains derived from it are, at least to

some extent, producing more haem relative to the other strains e.g. JCB387. The observation of the synthesis of *H. thermophilus* holocytochrome c_{552} in the absence of *ccm* genes, and its dependence on a haem precursor in *E. coli* JCB387, together imply that the only identified factor contributing to the formation of this cytochrome is availability of haem. This may be because this cytochrome c_{552} is highly thermostable [24] with the consequence that its apocytochrome has some tertiary structure, including a binding pocket into which haem inserts. The covalent attachment between apocytochrome and haem would then take place spontaneously. Furthermore, the insertion of haem may enhance the folding of apocytochrome c_{552} and thus can retard its degradation. This proposal can explain why the expression of holocytochrome c_{552} from the construct pKHC12ΔSig in the strain JCB387 was negligible unless the growth medium was supplemented with δ-aminolevulinic acid while the expression was normal from the same construct in the strain JCB712 without the supplementation with δ-aminolevulinic acid. The extra ten N-terminal amino acid residues might retard folding of apocytochrome c_{552} and thus the availability of haem may be crucial for displacing an equilibrium to a state with tertiary structure.

It may be that in the case of normal periplasmic cytochrome *c* assembly part of the biogenesis machinery is involved in holding the apocytochrome *c* and haem in the appropriate conformation, rather than in catalyzing the chemical reaction of thiol addition to vinyl groups of haem. Analysis of the recently released genome of *Helicobacter pylori* [25], which has both membrane bound and periplasmic cytochromes *c*, shows that it lacks nearly all the homologous genes for *c*-type cytochrome biogenesis identified in other studied Gram-negative organisms [26]. It has been suggested that since proteins responsible for disulfide bond formation are absent from this bacterium the problem of inevitable disulfide bond formation once an apocytochrome *c* carrying cysteines enters the periplasm is avoided [26]. Thus much of the *c*-type cytochrome biogenesis machinery, present in the periplasm, for reduction of disulfide is dispensable in *H. pylori*. This hypothesis can be compared with the maturation of cytochrome c_{562} in the cytoplasm of *E. coli*. Thermostable apocytochrome c_{552}, having some tertiary structure in the reducing environment of cytoplasm, does not need any other enzymatic assistance for the covalent attachment. The same may be true in the non-oxidising environment of the *H. pylori* periplasm. In vitro studies will eventually demonstrate the exact requirements for the maturation of this cytochrome c_{552}. Such studies are in progress. The biogenesis of thermophilic cytochrome c_{552} has not, however, been studied in *H. thermophilus*. We assume that the apocytochrome c_{552} is first translocated to the periplasm, the normal site of cytochrome c_{552} maturation in *H. thermophilus* [28], where the covalent attachment between apocytochrome c_{552} and haem takes place. There is no information available concerning cytochrome *c* biogenesis genes in *H. thermophilus* but we expect their presence following the pattern seen in either typical Gram-negative organisms such as *E. coli* or that in *H. pylori* [26]. At the elevated growth temperature of *H. thermophilus* we suspect that the periplasmic insertion of haem into its cytochrome c_{552} will need protein-mediated assistance. This is because the apo-protein will probably not spontaneously take up an appropriate three-dimensional structure for haem to attach covalently without assistance.

Acknowledgments

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